

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 14/62	A1	(11) International Publication Number: WO 98/02460 (43) International Publication Date: 22 January 1998 (22.01.98)
(21) International Application Number: PCT/DK97/00296 (22) International Filing Date: 4 July 1997 (04.07.97) (30) Priority Data: 0778/96 11 July 1996 (11.07.96) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventor; and (75) Inventor/Applicant (for US only): HANSEN, Louis, Brammer [DK/DK]; Bringebakken 3, DK-3500 Værløse (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo allé, DK-2880 Bagsvaerd (DK).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: SELECTIVE ACYLATION METHOD (57) Abstract A method of selectively acylating an insulin, an insulin analogue or a precursor thereof having a free ϵ -amino group of a Lys residue contained therein and at least one free α -amino group which method comprises reacting the ϵ -amino group with an activated amide in a polar solvent in the presence of a base.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

SELECTIVE ACYLATION METHOD**FIELD OF THE INVENTION**

The present invention relates to a method of introducing an acyl group into a peptide. More particularly, the invention relates to an improved method of acylating the ϵ -amino group of a lysine residue contained in a naturally occurring insulin or an analogue or a precursor thereof.

BACKGROUND OF THE INVENTION

Human insulin and closely related insulins have three primary amino groups in the molecule namely the α -amino groups of Gly^{A1} and Phe^{B1}, respectively, and the ϵ -amino group of Lys^{B29}. N-Acylation of an unprotected insulin may - depending on the conditions - lead to a complex mixture of mono-, di- and even triacylated products. However, although a certain preference for acylation of a specific position can often be observed the preference is not always sufficiently pronounced to make such direct acylation useful as a method of producing monoacylated insulins since the formation of the desired product may be accompanied by the formation of considerable amounts of closely related by-products. When by-products are formed, this happens at the expense of the desired product and may lead to problems in the purification of the desired product.

Acylation of only one or two specific amino groups in the insulin molecule can be achieved if a suitably protected intermediate is available. A suitable intermediate can be an insulin derivative in which the amino group(s) not to be acylated is (are) blocked with a protection group which can be removed selectively after the acylation has been performed. Such a protected intermediate can either be an insulin precursor or an insulin derivative in which it has been possible to introduce one or two protection groups in a specific way. For economic reasons, it is very attractive to avoid the use of specific of protection groups if possible.

Friesen HJ *et al.* (Semisynthetic Peptides and Proteins (Offord RE, DiBello C, eds.) 161-179, 1978, London) describe acylation of insulin with N-hydroxysuccinimide esters (in the following referred to as ONSu-esters) and other activated esters under various conditions, as summarised in tables 4 and 5 *l.c.* A certain selectivity is seen but invariably A1 monosubstituted and B29 monosubstituted products are obtained in mixtures with each other and with disubstituted products. The highest total yield of monosubstituted products reported is 75% but of this 85% was the A1 isomer. In other cases the total yield of monosubstituted products were in the range 45-55% of which 70-72% was the ϵ -B29 isomer.

As demonstrated by Friesen HJ *et al.* (in: Chemistry, Structure and Function of Insulin and Related Hormones, Proceedings of the Second International Insulin Symposium (Brandenburg D, Wollmer A, eds.), New York, 1980), the pH of the reaction medium has a very strong influence on the course of the reaction when insulin is acylated with ONSu-esters or certain other acylating agents. Thus, at pH 5-6 monoacylation preferably takes place in the B1 amino group and diacylated product will preferably be A1,B1-diacylated insulin. At pH 6.8-9.2 monoacylation preferably takes place in the A1 amino group while diacylated product can be either A1,B1- or A1, ϵ -B29-diacylated insulin. Finally, at pH above 10 monoacylation preferably takes place in the ϵ -amino group of Lys^{B29} while a further acyl group preferably goes to the amino group of A1.

A method which enables selective acylation of proinsulin, insulin or an insulin analogue having a free ϵ -amino group is disclosed in EP 0 712 861 A2 and EP 0 712 862 A2 (both Eli Lilly and Company). According to the method, unprotected insulins are acylated with soluble, activated fatty acid esters, in particular ONSu-esters, under basic conditions in a polar solvent.

EP 0 511 600 A2 (Kuraray Co., Ltd.) relates i.a. to protein derivatives of the formula [protein][Z]_n wherein [protein] represents a protein having a number of amino groups, [Z] is a residue represented by the formula -CO-W-COOH wherein W is a divalent long chain hydrocarbon group which may also contain certain hetero atoms and n represents an average of the number of amide bonds between [Z] and [protein]. The derivatives are prepared by reaction between the parent protein and a long chain carboxylic acid imide ester in an aqueous solution of a salt optionally also containing an organic solvent. No specificity of the acylation is mentioned and the fact that n is stated to be an average number seems to indicate that no specificity is achieved. It is mentioned, that insulin is one of the proteins from which derivatives according to the invention can be made, but no specific insulin derivative is disclosed in EP 0 511 600 nor is there any indication of a preferred [Z] or a preferred position in which [Z] should be introduced in order to obtain a useful insulin derivative.

Examples of insulin derivatives, acylated in the ϵ -amino group of the Lys residue contained therein, are described in WO 95/07931 (NOVO NORDISK A/S). The derivatives can be produced by acylation of the corresponding (A1,B1)-diBoc insulin, prepared from the corresponding insulin by reaction e.g. with di-*tert*-butyl dicarbonate and subsequently removing the protection groups and by acylation of a single chain insulin precursor which subsequently has to be processed further. Surprisingly, it has now been found that high yields of insulins, monoacylated in the ϵ -amino group of a Lys residue contained therein, can be obtained by the method of the present invention.

SUMMARY OF THE INVENTION

In its broadest aspect, the present invention relates to a method of selectively acylating an insulin, an insulin analogue or a precursor thereof having a free ϵ -amino group of a Lys
5 residue contained therein and at least one free α -amino group which method comprises reacting the ϵ -amino group with an activated amide in a polar solvent in the presence of a base.

DETAILED DESCRIPTION OF THE INVENTION

10 In a preferred embodiment, the parent insulin (*i.e.* the naturally occurring insulin, insulin analogue or insulin precursor to be acylated using the method according to the invention) is des(B30) human insulin. Examples of other preferred parent insulins are human insulin, porcine insulin and other naturally occurring insulins and also insulin analogues or insulin precursors in which at least one Lys having a free ϵ -amino group and an amino acid residue
15 having a free α -amino group is present.

One particularly preferred group of parent insulins are insulin analogues in which the amino acid residue in position B1 has been deleted.

20 Another particularly preferred group of parent insulins are insulin analogues which have Lys in position B28 and Pro in position B29. A preferred example from this group is Lys^{B28}Pro^{B29} human insulin.

Another particularly preferred group of parent insulins are insulin analogues which have Asp
25 in position B28. A preferred example from this group is Asp^{B28} human insulin.

Other particularly preferred groups of parent insulins are insulin analogues in which in which the A-chain and/or the B-chain have an N-terminal extension and insulin analogues in which the A-chain and/or the B-chain have a C-terminal extension.

30 Further insulin analogues which can be useful as parent insulins in the method of the present invention are such in which one or more of the amino acid residues, preferably one, two or three of them, has been substituted by another codable amino acid residue. Thus, in position A21 a parent insulin may in stead of Asn have an amino acid residue selected from
35 the group comprising Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Ser, Thr, Trp, Tyr or Val, in particular an amino acid residue selected from the group comprising Gly, Ala, Ser and Thr. Insulin analogues useful as parent insulins in the method of the present invention may also be modified by a combination of the changes outlined above.

By "insulin analogue" as used herein is meant a peptide having a molecular structure similar to that of human insulin including the disulphide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19} and an internal disulphide bridge between Cys^{A6} and Cys^{A11}, and which has insulin activity. When the amino acid at position B1 is deleted, the position of the remaining amino acids of the B-chain are not renumbered.

The expression "a codable amino acid residue" as used herein designates an amino acid residue which can be coded for by the genetic code, *i.e.* a triplet ("codon") of nucleotides.

The preferred parent insulin analogue des(B30) human insulin can be prepared by several methods, *i.a.* by enzymatic hydrolysis of human insulin, porcine insulin and insulin derivatives. Enzymes that facilitate this hydrolysis are lysyl endopeptidases, such as trypsin and *Achromobacter lyticus* proteases. Furthermore, des(B30) human insulin can be prepared in an enzymatic process from a single chain insulin precursor of the formula B(1-29)-Ala-Ala-Lys-A(1-21) wherein B(1-29) designates the amino acid sequence from position 1 to position 29 of the B-chain of human insulin and A(1-21) designates the amino acid sequence from position 1 to position 21 of the A-chain of human insulin. This precursor can be obtained as described in EP 163.529. A suitable enzyme for the process is *Achromobacter lyticus* protease 1, EC code No. 3.4.21.50. The enzyme can be used in a soluble form, or immobilised on a N-hydroxysuccinimide activated Sepharose. After completion of the reaction, the des(B30) human insulin formed can be isolated in a number of ways, *i.a.* by precipitation by addition of zinc or sodium salts.

The acylation reaction is usually carried out with a concentration of the parent insulin of from about 0.1% w/w to about 25% w/w, preferably from about 2% w/w to about 12% w/w, more preferred from about 3% w/w to about 8% w/w at the beginning of the reaction.

In one preferred embodiment of the invention, the acyl group to be introduced into the ϵ -amino group of a Lys residue is the acyl group of a monocarboxylic acid of the general formula (I):



wherein M is a long chain hydrocarbon group which may optionally be interrupted by one or more groups each independently selected from the group consisting of an oxygen atom and a sulphur atom. More preferred, the acyl group is the acyl group of an unbranched, aliphatic monocarboxylic acid having from 6 to 24 carbon atoms, in particular an acyl group selected

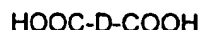
5

from the group comprising $\text{CH}_3(\text{CH}_2)_8\text{CO}-$, $\text{CH}_3(\text{CH}_2)_9\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{10}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{11}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{12}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{13}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{14}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{15}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{16}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{17}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{18}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{19}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{20}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{21}\text{CO}-$ and $\text{CH}_3(\text{CH}_2)_{22}\text{CO}-$.

5

In another preferred embodiment of the invention, the acyl group to be introduced into the ϵ -amino group of a Lys residue is one of the acyl groups of a dicarboxylic acid of the general formula (II):

10



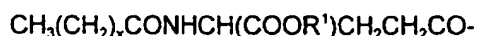
(II)

wherein D is a long chain hydrocarbon group which may optionally be interrupted by one or more groups each independently selected from the group consisting of an oxygen atom and a sulphur atom. More preferred, the acyl group is one of the acyl groups of a dicarboxylic acid of the general formula (II) wherein D is an unbranched, divalent aliphatic hydrocarbon group having from 6 to 22 carbon atoms, in particular an acyl group selected from the group comprising $\text{HOOC}(\text{CH}_2)_6\text{CO}-$, $\text{HOOC}(\text{CH}_2)_8\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{10}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{12}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{14}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{16}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{18}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{20}\text{CO}-$ and $\text{HOOC}(\text{CH}_2)_{22}\text{CO}-$.

20

In further preferred embodiment of the invention, the acyl group to be introduced into the ϵ -amino group of a Lys residue is a group of the general formula (III):

25



(III)

wherein x is an integer from 8 to 24 and R^1 is hydrogen or a group which can be exchanged with hydrogen after the acylation has been performed, for example methyl, ethyl or *tert*-butyl. Preferred values of x are 10, 12 and 14. When the acylation has been performed and R^1 is different from hydrogen, the ester group of which R^1 is a part can be hydrolysed to give the corresponding free acid and the alcohol R^1OH by methods known *per se*. Thus, when R^1 is methyl or ethyl, the hydrolysis can be performed under alkaline conditions and when R^1 is *tert*-butyl, the hydrolysis can be carried out using trifluoroacetic acid.

30

In further preferred embodiment of the invention, the acyl group to be introduced into the ϵ -amino group of a Lys residue is a group of the general formula (IV):

35



(IV)

wherein R^2 is hydrogen or a group which can be exchanged with hydrogen after the acylation has been performed, for example methyl, ethyl or *tert*-butyl. When the acylation has been performed and R^2 is different from hydrogen, the ester group of which R^2 is a part can be hydrolysed to give the corresponding free acid and the alcohol R^2OH by methods known *per se*. Thus, when R^2 is methyl or ethyl, the hydrolysis can be performed under alkaline conditions and when R^2 is *tert*-butyl, the hydrolysis can be carried out using trifluoroacetic acid.

The acylation of the parent insulin is carried out using an acylating agent which is an activated amide, more particularly an azolide of the acid corresponding to the acyl group to be introduced. Such azolides (1-acylazoles) can be prepared according to known methods, see for example Staab HA *Angew. Chem.* **74** (1962) 407-423. Examples of azoles which can be used in the preparation of the acylating agents of this invention are pyrazole, imidazole, 1,2,3-triazole, 1,2,4-triazole, tetrazole, phenyltetrazole and - where possible - the corresponding benzanelated compounds e.g. 1H-indazol, benzimidazole and benzotriazole. Preferred azoles for the preparation of the acylating agent are 1,2,4-triazole, benzotriazole and substituted benzotriazoles. Optionally, the azoles mentioned can be substituted with one or more substituents selected from the group comprising alkyl ($C_1 - C_5$, branched or unbranched, in particular methyl, ethyl, propyl and isopropyl), halogen (e.g. fluoro, chloro and bromo, in particular fluoro and chloro), nitro, alkoxy ($C_1 - C_5$, branched or unbranched, in particular methoxy, ethoxy, propoxy and isopropoxy), dialkylated amino ($C_1 - C_5$, branched or unbranched), sulphonic acid, carboxy and alkoxycarbonyl ($C_1 - C_5$, branched or unbranched). A preferred group of acylating agents are 1-acyl benzotriazoles and a preferred acylating agent is 1-tetradecanoyl benzotriazole. Other preferred 1-acyl benzotriazoles are such which are derived from mono- or disubstituted benzotriazoles, e.g. 5-substituted or 6-substituted or 5,6-disubstituted benzotriazoles such as 5-methylbenzotriazole, 5-chlorobenzotriazole, 6-chlorobenzotriazole, 5-nitrobenzotriazole, 5,6-dimethylbenzotriazole and 5,6-dichlorobenzotriazole.

The acylation is carried out in a polar solvent which can be a mixture of water and at least one water-miscible organic solvent. Examples of water-miscible solvents which may be useful either alone or in mixtures are lower alcohols, e.g. methanol, ethanol and 2-propanol, lower straight chain and branched ketones, e.g. acetone, cyclic ethers, e.g. tetrahydrofuran and dioxane, straight chain and cyclic amides, e.g. dimethylformamide, dimethylacetamide and N-methyl-2-pyrrolidone, nitriles e.g. acetonitrile and sulfoxides, e.g. dimethyl sulfoxide. Most preferred among the water-miscible organic solvents are N-methyl-2-pyrrolidone, dimethylformamide, dimethylacetamide and dimethyl sulfoxide.

The amount of water in the reaction medium may vary within a broad range so that the medium in general contains from about 1% w/w to about 99% w/w of water. When N-methyl-2-pyrrolidone is used as the water-miscible organic solvent the amount of water in the medium is preferably from about 1% w/w to about 90% w/w, more preferred from about 10% w/w or about 20% w/w to about 75% w/w.

The acylation is carried out in the presence of an excess of a medium strong or strong base which can be either an inorganic base or an organic base. Examples of inorganic bases are sodium hydroxide, potassium hydroxide, sodium carbonate and potassium carbonate. Examples of organic bases are tertiary amines, e.g. ethyldiisopropylamine, triethylamine and diethylethanolamine. Preferably, an organic base is used. The amount of base used is generally in excess of 5 mol per mol of parent insulin, e.g. 10 to 30 mol or even 100 mol of base per mol of parent insulin.

Basically, the lower limit of the temperature at which the acylation can be carried out is determined by the freezing point of the medium while the upper limit is determined by the temperature at which the parent insulin or the acylated insulin will deteriorate. This again will depend *i.a.* on the composition of the medium. Thus, while it may be possible to carry out the reaction at temperatures between -30 °C and 70 °C it is usually most convenient to carry out the reaction at a temperature between about -5 °C and about 30°C.

Isolation and purification of the products prepared by the method of the present invention can be carried out by methods known *per se*, including gel filtration and chromatography.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, either separately or in any combination thereof, be material for realising the invention in diverse forms thereof.

EXAMPLES

Analytical

The reaction mixtures - except in Example 4 - were analysed by RP-HPLC as described below using a Merck/Hitachi apparatus equipped with a Diode Array Detector.

Column: 4.0 x 125 mm, operated at 60°.

Column material: dimethyl butyl dimethyl silyl substituted 100 Å, 5 mm silica.

Eluent gradient system:

5 Eluent A: acetonitrile (7.8%) in water containing ammonium sulphate (2.0%) at pH 2.5.

Eluent B: acetonitrile (53.9%) in water.

Flow rate: 1.0 ml/min.

Detection: UV absorption at 280 nm.

Cycle time: 65 min.

10 The yields stated are based on the area of the peaks in the chromatograms.

The identity of the acylated insulins was determined by their retention time in RP-HPLC and by isolation and mass spectrometry /peptide mapping performed on the acylated products 1) without any pre-treatment, 2) after pre-treatment with dithiotreitol for reduction of the disulphide bonds and 3) after pre-treatment with dithiotreitol and *Staphylococcus aureus* protease V8 for cleavage of the B-chain between position 21 and position 22.

Preparation of starting materials:

20 **EXAMPLE 1**

Preparation of 1-tetradecanoylbenzotriazole.

25 Benzotriazole (59.6 g, 0.50 mol) was dissolved in *tert*-butyl methyl ether (950 ml) at 20° and triethylamine (50.6 g, 0.50 mol) was added. Tetradecanoyl chloride (125.0 g, 0.51 mol) was added over a period of 30 min, the temperature being kept at 20-30°C by cooling. The resulting precipitate was removed by filtration, and the filtrate evaporated to dryness under reduced pressure at 60°C. The residue was dissolved in acetone (375 ml) at 60°C. After cooling to 0°C, the resulting crystals were isolated by filtration, washed with acetone (120 ml) and dried to constant weight under reduced pressure at 20°C.

30 Yield: 142.3 g (86%) of white crystalline 1-tetradecanoylbenzotriazole, melting at 58.0°C (peak value) determined by Differential Scanning Calorimetry (DSC).

Under similar reaction conditions, the following acylated triazoles were also prepared:

35 1-tetradecanoyl-1,2,4-triazole, DSC: 59.7°C (recryst. from tetrahydrofuran),
5-methyl-1-tetradecanoylbenzotriazole, DSC: 69.5°C (recryst. from 2-propanol),

5-chloro-1-tetradecanoylbenzotriazole (NMR data seem to indicate that the product also contained some 6-chloro-1-tetradecanoylbenzotriazole), DSC: 52.1°C (recryst. from 2-propanol),

5-nitro-1-tetradecanoylbenzotriazole, DSC: 117.5°C (recryst. from acetone),

5 5,6-dichloro-1-tetradecanoylbenzotriazole, DSC: 60.6°C (recryst. from tetrahydrofuran),

5,6-dimethyl-1-tetradecanoylbenzotriazole, DSC: 61.6°C (recryst. from acetone),

1-dodecanoylbenzotriazole, DSC: 43.9°C (recryst. from n-heptane) and

1-hexadecanoylbenzotriazole, DSC 62.4°C (recryst. from *tert*-butyl acetate).

10 EXAMPLE 2

Preparation of 1-(19-carboxynonadecanoyl)benzotriazole.

15 A solution of eicosanedioic acid (3.43 g, 10.0 mmol), dicyclohexylcarbodiimide (2.06 g, 10.0 mmol), benzotriazole (1.19 g, 10.0 mmol) and dimethylamino-pyridine hydrochloride in anhydrous tetrahydrofuran (200 ml) was stirred for two days at room temperature. The resulting suspension was filtered, and the filtrate evaporated to dryness under reduced pressure. The residue was suspended in dichloromethane (100 ml), and undissolved material was isolated by filtration. The dried filter cake (2.0 g) was recrystallised from acetone (200 ml) yielding 0.70 g (16%) of white crystalline 1-(19-carboxynonadecanoyl)benzotriazole, melting at 111.9°C (DSC peak value).

EXAMPLE 3

Preparation of 1-(17-carboxyheptadecanoyl)benzotriazole.

25 Under similar reaction conditions as in Example 2, but using octadecanedioic acid in stead of eicosanedioic acid, 1-(17-carboxyheptadecanoyl)-benzotriazole was prepared. The compound had a melting point of 105.2°C (DSC peak value).

30 EXAMPLE 4

Preparation of des(B30) human insulin from a single chain insulin precursor.

35 Single chain insulin precursor of the formula B(1-29)-Ala-Ala-Lys-A(1-21) wherein B(1-29) designates the amino acid sequence from position 1 to position 29 of the B-chain of human insulin and A(1-21) designates the amino acid sequence from position 1 to position 21 of the A-chain of human insulin was obtained as described in EP 163.529.

10

- 5 g of wet salt cake containing about 1.5 g of the above-mentioned single chain precursor was dissolved in 25 mM sodium hydrogen carbonate (350 ml) at room temperature. The pH value of the solution was adjusted to 9.0 by addition of 1.0 M sodium hydroxide (1 ml). *Achromobacter lyticus* protease 1, immobilised on N-hydroxysuccinimide activated Sepharose (10 ml of gel with an enzyme density of approximately 1.0 mg/l) was added. The reaction mixture was stirred for 23 hours at room temperature and the immobilised enzyme was then removed by filtration. The filtrate was analysed by RP-HPLC as described below.
- Based on the area of the peaks in the chromatogram, the relative amount of des(B30) human insulin found in the reaction mixture was 95.2% (retention time: 27.7 min) and the relative amount of starting material was 0.1% (retention time: 15.0 min).

Analytical

- The reaction mixture was analysed by RP-HPLC on equipment supplied by Waters.

Column: 4.0 x 250 mm, operated at 50°.

Column material: YMC 120 A, 5 my OdDMe silylated silica gel.

Eluent gradient system:

- Eluent A: 0.20 M sodium sulphate, 0.04 M phosphoric acid in acetonitrile (10%) in water, pH 2.3.

Eluent B: acetonitrile (50%) in water.

Run time: 60 minutes

Flow rate: 1.0 ml/min

- Detection: UV absorption at 214 nm

gradient: 00.00 min 64% A + 36% B
 40.00 min 40% A + 60% B
 41.00 min 64% A + 36% B

- Preparation of acylated insulins:**

EXAMPLE 5

Synthesis of N^{C^{B29}}-tetradecanoyl des(B30) human insulin.

- Des(B30) human insulin (0.50 g, assay: about 80%~0.070 mmol) was dissolved in N-methyl-2-pyrrolidone (7.0 ml) and water (3.5 ml) at 20°C. The solution was cooled to 0°C. Triethylamine (0.40 ml) was added. 1-Tetradecanoylbenzotriazole (1.50 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added over a period of 30 minutes. The turbid

reaction mixture was stirred at 0°C for 30 minutes, then at 20°C for an additional 30 minutes.

The reaction mixture was analysed by RP-HPLC with the following results:

5

Retention time, min	Compound	% yield
11.00	starting material	7.3
25.20	N ^{A1} -tetradecanoyl des(B30) human insulin	1.8
29.66	N ^{C29} -tetradecanoyl des(B30) human insulin	71.0
49.85	Diacylated des(B30) human insulin	7.7
>50	unknown products totalling	10.4

EXAMPLE 6

10 Synthesis of N^{C29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (0.50 g, assay: about 66%~ 0.58 mmol) was dissolved in N-methyl-2-pyrrolidone (7.5 ml) and water (1.0 ml) at 20°C. Triethylamine (0.40 ml) was added. 1-Tetradecanoyl-1,2,4-triazole (1.0 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The homogenous reaction mixture was stirred at 20°C for 10 minutes and then analysed by RP-HPLC with the following results:

15

Retention time, min	Compound	% yield
11.43	starting material	30.0
25.89	N ^{A1} -tetradecanoyl des(B30) human insulin	8.4
30.37	N ^{C29} -tetradecanoyl des(B30) human insulin	43.8
50.20	Diacylated des(B30) human insulin	8.7
>50.20	unknown products totalling	3.8

20

EXAMPLE 7

Synthesis of N^{C29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (0.50 g, assay: about 80%~0.070 mmol) was dissolved in dimethyl sulfoxide (7.0 ml) and water (3.5 ml) at 20°C and triethylamine (0.40 ml) was added. 1-Tetradecanoylbenzotriazole (1.80 ml of a 0.10 M solution in N-methyl-2-

25

pyrrolidone) was added over a period of 2 minutes. The turbid reaction mixture was stirred at 20°C for 90 minutes. The resulting homogeneous reaction mixture was analysed by RP-HPLC showing a yield of 59.4% N^{tB29}-tetradecanoyl des(B30) human insulin.

5 EXAMPLE 8

Synthesis of N^{tB29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (0.50 g, assay: about 80%~0.070 mmol) was dissolved in dimethylformamide (7.0 ml) and 6M aqueous urea (2.0 ml) at 20°C. The solution was
10 cooled to 0°C, and triethylamine (0.40 ml) was added. 1-Tetradecanoylbenzotriazole (1.10 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The turbid reaction mixture was stirred at 0°C for 60 minutes. The resulting slightly turbid reaction mixture was analysed by RP-HPLC showing a yield of 74.2% N^{tB29}-tetradecanoyl des(B30) human insulin.

15

EXAMPLE 9

Synthesis of N^{tB29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (0.50 g, assay: about 80%~0.070 mmol) was dissolved in N-methyl-2-pyrrolidone (7.0 ml) and water (2.0 ml) at 20°C. The solution was cooled to 0°C.
20 Triethylamine (0.20 ml) was added. 1-Tetradecanoylbenzotriazole (1.00 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The turbid reaction mixture was stirred at 0°C for 65 minutes. The resulting reaction mixture was analysed by RP-HPLC showing a yield of 68.3% N^{tB29}-tetradecanoyl des(B30) human insulin.

25

EXAMPLE 10

Synthesis of N^{tB29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (0.50 g, assay: about 80%~0.070 mmol) was dissolved in N-methyl-2-pyrrolidone (7.0 ml) and 3M aqueous urea (3.5 ml) at 20°C.
30 Ethyldiisopropylamine (0.50 ml) was added. 1-Tetradecanoylbenzotriazole (1.50 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added over a period of 2 minutes. The turbid reaction mixture was stirred at 20°C for 90 minutes. The resulting reaction mixture was analysed by RP-HPLC showing a yield of 61.9% N^{tB29}-tetradecanoyl des(B30) human
35 insulin.

EXAMPLE 11

Synthesis of N^{B29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (5.0 g, assay: about 34%~0.30 mmol) was dissolved in N-methyl-
5 2-pyrrolidone (20.0 ml) at 20°C. After cooling to 0°C, triethylamine (1.50 ml) was added. 5-Nitro-1-tetradecanoylbenzotriazole (3.20 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added over a period of 10 minutes. The reaction mixture was stirred at 0°C for 30 minutes. The resulting reaction mixture was analysed by RP-HPLC showing a yield of 58.3% N^{B29}-tetradecanoyl des(B30) human insulin.

10

EXAMPLE 12

Synthesis of N^{B29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (5.0 g, assay: about 34%~0.30 mmol) was dissolved in N-methyl-
15 2-pyrrolidone (20.0 ml) at 20°C. After cooling to 0°C water (7.5 ml) and triethylamine (1.50 ml) was added. 5-Chloro-1-tetradecanoylbenzotriazole (4.5 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added over a period of 15 minutes. The turbid reaction mixture was stirred at 0°C for 3 hours. The resulting reaction mixture was analysed by RP-HPLC showing a yield of 77.7% N^{B29}-tetradecanoyl des(B30) human insulin.

20

EXAMPLE 13

Synthesis of N^{B29}-tetradecanoyl human insulin.

Human insulin (50 mg, 0.009 mmol) was dissolved in N-methyl-2-pyrrolidone (0.7 ml) and
25 water (0.35 ml) at 0°C. Triethylamine (0.040 ml) was added. 1-Tetradecanoylbenzotriazole (0.150 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The turbid reaction mixture was stirred at 0°C for 150 minutes. The resulting homogeneous reaction mixture was analysed by RP-HPLC showing a yield of 64.0% N^{B29}-tetradecanoyl human insulin (retention time: 27.75 min).

30

EXAMPLE 14

Synthesis of N^{B29}-tetradecanoyl porcine insulin.

Porcine insulin (50 mg, 0.009 mmol) was dissolved in N-methyl-2-pyrrolidone (0.7 ml) and
35 water (0.35 ml) at 0°C. Triethylamine (0.040 ml) was added. 1-Tetradecanoylbenzotriazole (0.150 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The turbid reaction mixture was stirred at 0°C for 155 minutes. The resulting

homogeneous reaction mixture was analysed by RP-HPLC showing a yield of 62.5% N^{B29}-tetradecanoyl porcine insulin (retention time: 28.99 min).

EXAMPLE 15

- 5 Synthesis of N^{B29}-(19-carboxynonadecanoyl) des(B30) human insulin.
-

Des(B30) human insulin (5.0 g, assay: about 34%~0.30 mmol) was dissolved in N-methyl-2-pyrrolidone (30.0 ml) and water (6.9 ml) at 20°C. Triethylamine (1.10 ml) was added. 1-(19-carboxynonadecanoyl)benzotriazole (5.0 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The reaction mixture was stirred at 20°C for 4.5 hours. The resulting reaction mixture was analysed by RP-HPLC showing a yield of 30.0% N^{B29}-(19-carboxynonadecanoyl) des(B30) human insulin (retention time: 32.19 min). The amount of starting material was 57.3%.

15 **EXAMPLE 16**

Synthesis of N^{B29}-(17-carboxyheptadecanoyl) des(B30) human insulin.

Under similar reaction conditions as in Example 15, but using 1-(17-carboxyheptadecanoyl)benzotriazole in stead of 1-(19-carboxynonadecanoyl)benzotriazole, the resulting reaction mixture was analysed by RP-HPLC showing a yield of 24.9% N^{B29}-(17-carboxyheptadecanoyl) des(B30) human insulin (retention time: 26.41 min). The amount of starting material was 68.3%.

EXAMPLE 17

- 25 Synthesis of N^{B29}-tetradecanoyl des(B30) human insulin.
-

Des(B30) human insulin (0.50 g, assay: about 80%~0.070 mmol) was dissolved in N-methyl-2-pyrrolidone (7.0 ml) and 3M aqueous urea (3.5 ml) at 20°C. Triethylamine (0.40 ml) was added, and the solution was cooled to -13°C. 1-Tetradecanoylbenzotriazole (1.80 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The resulting suspension was stirred at -13°C for 47 hours. The reaction mixture was analysed by RP-HPLC showing a yield of 65.2% N^{B29}-tetradecanoyl des(B30) human insulin.

EXAMPLE 18

- 35 Synthesis of N^{B29}-tetradecanoyl des(B30) human insulin.
-

Des(B30) human insulin (0.50 g, assay about 80%~0.070 mmol) was dissolved in N-methyl-2-pyrrolidone (7.0 ml) and water (2.0 ml) at 20°C. The solution was cooled to 0°C.

15

Triethylamine (0.40 ml) was added. 5-Methyl-1-tetradecanoylbenzotriazole (1.00 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The turbid reaction mixture was stirred at 0°C for 2 hours. The resulting solution was analysed by RP-HPLC showing a yield of 66.8% N^{tB29}-tetradecanoyl des(B30) human insulin.

5

EXAMPLE 19

Synthesis of N^{tB29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (0.50 g, assay about 80%~0.070 mmol) was dissolved in N-methyl-2-pyrrolidone (7.0 ml) and water (2.0 ml) at 20°C. Triethylamine (0.40 ml) was added. 5,6-Dichloro-1-tetradecanoylbenzotriazole (1.10 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added in one portion. The turbid reaction mixture was stirred at 20°C for 1 hours. The resulting solution was analysed by RP-HPLC showing a yield of 68.8% N^{tB29}-tetradecanoyl des(B30) human insulin.

15

EXAMPLE 20

Synthesis of N^{tB29}-tetradecanoyl des(B30) human insulin.

Des(B30) human insulin (5.0 g, assay about 30%~0.26 mmol) was dissolved in N-methyl-2-pyrrolidone (30.0 ml) and water (6.0 ml) at 20°C. Triethylamine (1.08 ml) was added. 5,6-Dimethyl-1-tetradecanoylbenzotriazole (4.00 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added over a period of 15 minutes. The turbid reaction mixture was stirred at 20°C for 1 hours. The resulting solution was analysed by RP-HPLC showing a yield of 54.6% N^{tB29}-tetradecanoyl des(B30) human insulin.

25

EXAMPLE 21

Synthesis of N^{tB29}-dodecanoyl des(B30) human insulin.

Des(B30) human insulin (0.10 g, assay about 80%~0.014 mmol) was dissolved in N-methyl-2-pyrrolidone (1.4 ml) and water (0.4 ml) at 20°C. The solution was cooled to 0°C. Triethylamine (0.08 ml) was added. 1-dodecanoylbenzotriazole (0.20 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added over a period of 3 minutes. The turbid reaction mixture was stirred at 0°C for 40 minutes. The resulting solution was analysed by RP-HPLC showing a yield of 58.9% N^{tB29}-dodecanoyl des(B30) human insulin (retention time: 23.17 minutes), and 23.7% of starting material.

35

EXAMPLE 22

Synthesis of N^{tB29}-hexadecanoyl des(B30) human insulin.

Des(B30) human insulin (0.10 g, assay about 80%~0.014 mmol) was dissolved in N-methyl-2-pyrrolidone (1.4 ml) and water (0.4 ml) at 20°C. The solution was cooled to 0°C. Triethylamine (0.08 ml) was added. 1-hexadecanoylbenzotriazole (0.20 ml of a 0.10 M solution in N-methyl-2-pyrrolidone) was added over a period of 3 minutes. The turbid reaction mixture was stirred at 0°C for 60 minutes. The still turbid reaction mixture was analysed by RP-HPLC showing a yield of 32.6% N^{tB29}-hexadecanoyl des(B30) human insulin (retention time: 33.41 minutes), and 59.2% of starting material.

CLAIMS

1. A method of selectively acylating an insulin, an insulin analogue or a precursor thereof having a free ϵ -amino group of a Lys residue contained therein and at least one free α -amino group which method comprises reacting the ϵ -amino group with an activated amide in a polar solvent in the presence of a base.
2. The method of claim 1 wherein the insulin is human insulin.
3. The method of claim 1 wherein the insulin is porcine insulin.
4. The method of claim 1 wherein the parent insulin is an insulin analogue.
5. The method of claim 4 wherein the parent insulin is des(B30) human insulin.
6. The method of claim 4 wherein the insulin has Lys in position B28 and Pro in position B29.
7. The method of claim 4 wherein the insulin is an insulin analogue in which Phe^{B1} has been deleted.
8. The method of claim 4 wherein the insulin is an insulin analogue in which the A-chain and/or the B-chain has an N-terminal extension.
9. The method of claim 4 wherein the insulin is an insulin analogue in which the A-chain and/or the B-chain has an C-terminal extension.
10. The method of according to any one of the preceding claims wherein the acyl group to be introduced is the acyl group of a monocarboxylic acid of the general formula:
- $$\text{M-COOH} \quad (I)$$
- wherein M is a long chain hydrocarbon group which may optionally be interrupted by one or more groups each independently selected from the group consisting of an oxygen atom and a sulphur atom.
11. The method of claim 10 wherein the acyl group to be introduced is the acyl group of an unbranched, aliphatic monocarboxylic acid having from 6 to 24 carbon atoms.

18

12. The method of claim 10 wherein the acyl group to be introduced is selected from the group comprising $\text{CH}_3(\text{CH}_2)_8\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{10}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{12}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{14}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{16}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{18}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{20}\text{CO}-$ and $\text{CH}_3(\text{CH}_2)_{22}\text{CO}-$.

5 13. The method of claim 12 wherein the acyl group to be introduced is $\text{CH}_3(\text{CH}_2)_{12}\text{CO}-$.

14. The method according to any one of the claims 1-9 wherein the acyl group to be introduced is one of the acyl groups of a dicarboxylic acid of the general formula:

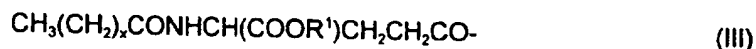


wherein D is a long chain hydrocarbon group which may optionally be interrupted by one or more groups each independently selected from the group consisting of an oxygen atom and a sulphur atom.

15 15. The method of claim 14 wherein the acyl group to be introduced is one of the acyl groups of a dicarboxylic acid of the general formula (II) wherein D is an unbranched, divalent aliphatic hydrocarbon group having from 6 to 22 carbon atoms.

20 16. The method of claim 14 wherein the acyl group to be introduced is selected from the group comprising $\text{HOOC}(\text{CH}_2)_4\text{CO}-$, $\text{HOOC}(\text{CH}_2)_6\text{CO}-$, $\text{HOOC}(\text{CH}_2)_8\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{10}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{12}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{14}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{16}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{18}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{20}\text{CO}-$ and $\text{HOOC}(\text{CH}_2)_{22}\text{CO}-$.

25 17. The method according to any one of the claims 1-9 wherein the acyl group to be introduced is a group of the general formula (III):



30 wherein x is an integer from 8 to 24 and R^1 is hydrogen or a group which can be exchanged with hydrogen when the acylation has been performed.

18. The method of claim 17, wherein x is 10, 12 or 14.

35 19. The method of claim 17, wherein R^1 is methyl, ethyl or *tert*-butyl.

20. The method according to any one of the claims 1-9 wherein the acyl group to be introduced is a group of the general formula (IV):



5 wherein R² is hydrogen or a group which can be exchanged with hydrogen when the acylation has been performed.

21. The method of claim 20, wherein R² is methyl, ethyl or *tert*-butyl.

10 22. The method according to any one of the preceding claims, wherein the acylation is carried out using an acylating agent which is an azolide of the acid corresponding to the acyl group to be introduced.

15 23. The method of claim 22, wherein the azolide is derived from an azole selected from the group comprising pyrazole, imidazole, 1,2,3-triazole, 1,2,4-triazole, tetrazole and phenyl-tetrazole.

24. The method of claim 22, wherein the azolide is derived from a benzannelated azole.

20 25. The method of claim 22, wherein the azolide is derived from an azole selected from the group comprising indazole, benzimidazole and benzotriazole.

26. The method of claim 22, wherein the acylating agent is 1-tetradecanoyl benzotriazole.

25 27. The method of claim 22, wherein the azolide is derived from a benzotriazole which is mono- or disubstituted with a substituent selected from the group comprising C₁-C₄ alkyl, halogen and nitro.

30 28. The method of claim 27, wherein the azolide is derived from the group comprising 5-methylbenzotriazole, 5-chlorobenzotriazole, 5-nitrobenzotriazole, 5,6-dimethylbenzotriazole and 5,6-dichlorobenzotriazole.

35 29. The method according to any one of the preceding claims, wherein the organic solvent is selected from the group comprising N-methyl-2-pyrrolidone, dimethylformamide, dimethylacetamide and dimethyl sulfoxide.

30. The method according to any one of the preceding claims, wherein the medium contains from about 1% w/w to about 99% w/w of water.

20

31. The method according to any one of the preceding claims, wherein the solvent is N-methyl-2-pyrrolidone containing from about 1% w/w to about 90% w/w, preferably from about 20% w/w to about 75% w/w of water.

5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00296

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, BIOSIS, WPI, CLAIMS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0712861 A2 (ELI LILLY AND COMPANY), 22 May 1996 (22.05.96), page 4, line 5 - line 30; page 5, line 28 - line 50 --	1-31
X	EP 0712862 A2 (ELI LILLY AND COMPANY), 22 May 1996 (22.05.96), page 2, line 6 - line 9; page 3, line 40 - line 57, examples, claims --	1-31
A	Dialog Information Services, file 351, DERWENT WPI, Dialog accession no. 008078156, WPI accession no. 89-343268/198947, KODAMA KK: "Insulin derivs. useful as antidiabetic drugs - in which fatty acids are bonded to amino gps. of B1 and/or B29-amino-acids in B-chain of insulin"; JP,A,1254699, 19891011 --	1-31

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

10 November 1997

14.11.97

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Anneli Jönsson
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00296

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 9507931 A1 (NOVO NORDISK A/S), 23 March 1995 (23.03.95), example 9-10, 16-19, claims</p> <p style="text-align: center;">-- -----</p>	1-31

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00296

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
EP	0712861	A2	22/05/96	AU	4237396 A	17/06/96
				FI	972031 A	14/05/97
				IL	115973 D	00/00/00
				NO	972169 A	12/05/97
				WO	9615804 A	30/05/96

EP	0712862	A2	22/05/96	AU	4237296 A	17/06/96
				IL	115972 D	00/00/00
				NO	972185 A	13/05/97
				US	5646242 A	08/07/97
				WO	9615803 A	30/05/96

WO	9507931	A1	23/03/95	AU	7652094 A	03/04/95
				BG	100420 A	31/12/96
				BR	9407508 A	07/01/97
				CA	2171424 A	23/03/95
				CN	1133598 A	16/10/96
				CZ	9600789 A	16/10/96
				EP	0792290 A	03/09/97
				FI	961220 A	14/05/96
				HU	75991 A	28/05/97
				HU	9600676 D	00/00/00
				IL	110977 D	00/00/00
				JP	9502867 T	25/03/97
				NO	961070 A	15/05/96
				PL	313444 A	08/07/96
				SK	32496 A	06/11/96
				ZA	9407187 A	17/03/95
